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Mutations in the *csgD* Promoter Associated with Variations in Curli Expression in Certain Strains of *Escherichia coli* O157:H7

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Single-base-pair *csgD* promoter mutations in human outbreak *Escherichia coli* O157:H7 strains ATCC 43894 and ATCC 43895 coincided with differential Congo red dye binding from curli fiber expression. Red phenotype *csgD*::*lacZ* promoter fusions had fourfold-greater expression than white promoter fusions. Cloning the red variant *csgDEFG* operon into white variants induced the red phenotype. Substrate utilization differed between red and white variants.

Many *Escherichia coli* organisms and salmonellae express coiled surface appendages, known as curli fibers and thin, aggregegative fimbriae, respectively, that are typically produced under stressful environmental conditions, such as low temperature, low osmolarity, and stationary growth (3, 9, 10). Curli fibers bind fibronectin, laminin, certain serum proteins, and Congo red dye (4, 8, 9, 18). Two divergently transcribed operons are required for curli expression: *csgBA* encodes the curli subunit protein (CsgA) and a nucleator protein (CsgB); *csgDEFG* encodes a transcriptional regulator (CsgD), an outer membrane lipoprotein (CsgG), and two putative curli assembly factors (CsgE and CsgF). Transcription from the *csgBA* promoter requires *csgD* expression; both operons require stationary-phase sigma factor (σ^s) for expression (1, 4, 10). Expression of thin, aggregative fimbriae in *Salmonella enterica* serovar Typhimurium is regulated by a similar *agf* operon (13).

Curli expression has not been reported for enterohemorrhagic *E. coli* (EHEC) O157:H7, the most common Shiga-toxigenic serotype associated with human disease (11). In order to identify potential factors involved in this pathogen's survival and persistence outside of the mammalian host, we screened 49 diverse bovine and human *E. coli* O157:H7 isolates for curli expression on Congo red indicator (CRI) plates after 48 h at 28°C (5). The 41 bovine isolates were from infected beef calves in five states (6). The eight human-associated isolates were American Type Culture Collection (ATCC, Rockville, Md.) strains ATCC 35150, ATCC 43888, ATCC 43889, ATCC 43890, ATCC 43894, and ATCC 43895 and Washington state strains Tarr4A and Tarr1A (2, 19). All of the bovine and six of the human isolates displayed smooth, moist, white colonies typical of the curli-deficient *E. coli* strain HB101 on CRI plates (9). However, strains ATCC 43894 and ATCC 43895 displayed mixed red and white colonies. Red colonies were dry, rough, and curliated as verified by electron microscopy (results not shown). Red and white colonies retained their parental phenotypes when subcultured on CRI plates with or without 1% NaCl and at either 28 or 37°C, suggesting that curli expression was neither low-temperature nor low-osmolarity dependent. Red variants passaged daily (1:100) in Luria-Bertani broth (Difco Laboratories, Detroit, Mich.) at 37°C generated mixed red and white phenotypes in as few as 3 passages, with white variants persisting at 40 to 60% of total colonies over 10 passages. White variants were stable under all growth conditions tested except for one, strain ATCC 43894 (stored frozen for 6 months at −80°C in brain heart infusion broth with 15% glycerol), which generated rare (10^{-4}) red variants. These findings suggest that curli expression by *E. coli* O157:H7 strains is uncommon and/or unstable.

Red and white colonies derived from CRI plate passage of strains ATCC 43895 and ATCC 43894 (n = 16) (Fig. 1), Tarr1A, Tarr4A, and two randomly selected bovine isolates (strains 84-2 and 161-2) were further analyzed. The *csgBA* and *csgDEFG* operons from these 20 isolates were PCR amplified and compared by gel electrophoresis (15). Primers PROfor (5′-CAAGAGAGCTGTCGCCTGC) and CDrev (5′-CAACT TCGTCAAAGCAATGGG) amplified the *csgBA* operons; primers COfor (5′-GCTTAAACAGTAAAATGCCGG) and PROrev (5′-CTAAATCATAACCTGCTGCGG) amplified the *csgDEFG* operons. The product size matched the predicted *E. coli* K-12 sequence (GenBank accession no. X90754), indicating that the lack of curli expression in white variants was not due to large DNA deletions or insertions (results not shown). The amplified *csgBA* operon of strain 43895OR was sequenced (GenBank accession no. AF275733), and the predicted CsgB protein had 100% identity to *E. coli* K-12 CsgB over 151 amino acids. However, CsgA (minus the leader peptide) had only 96% identity to K-12 CsgB over 132 amino acids and contained an extra glycine at position 10 of the deduced sequence. Thus, *E. coli* O157:H7 and K-12 curli fibers may be structurally distinct.

The *csgB*-to-*csgD* intergenic region of all 16 ATCC 43894 and ATCC 43895 strain variants was amplified using primers PROfor and PROrev. Sequence comparison revealed single-base-pair differences at either base −7 or −9 from the putative *csgD* transcriptional start (Fig. 2). All white variants contained thymine at base −7 and guanine at base −9, matching *E. coli* K-12. However, ATCC 43894 red variants had adenine at base

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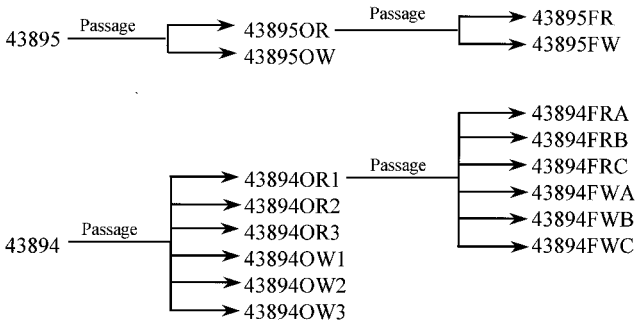

FIG. 1. Derivation of red (curliated) and white (noncurliated) phenotypic variants of EHEC O157:H7 strains ATCC 43895 and ATCC 43894. Strain designations containing "R" or "W" in the last or next to last position indicate colony phenotype as red or white, respectively. To induce passage, a single colony was inoculated into Luria-Bertani broth (16 h, 37°C), and then a 1:100 dilution was grown in fresh Luria-Bertani broth (16 h, 37°C) for three successive days, followed by the plating of diluted Luria-Bertani broth onto CRI plates (48 h, 28°C).

−7 and ATCC 43895 red variants had thymine at base −9. *E. coli* K-12 *csgDEFG* transcription initiates at position −148 from the *csgD* start codon and possesses features typical of an σ^{70}-dependent promoter (4). Compared to the consensus σ^{70}-dependent −10 promoter sequence (5′-TATAAT) (12), K-12 and EHEC O157 white variants differed at two positions (5′-TAGATT). However, red variant strain sequences of ATCC 43895 (5′-TATATT) and ATCC 43894 (5′-TAGAAT) differed at only one position. The greater identity of the −10 sequence of red variants to the consensus σ^{70}-dependent promoter could enhance recognition of the σ^{70}-dependent RNA polymerase, resulting in the red-to-white phenotype switch. However, recognition and use of σ^{S} at this site cannot be excluded, nor can we exclude contributions from undetermined red phenotype mutations. In serovar Typhimurium, a nucleotide transversion at position −44 from the *agfD* transcriptional start and an insertion between the −10 and −35 regions both resulted in expression of stable, thin, aggregative fimbriae (14). In contrast, we found *E. coli* O157:H7 transversions within the putative −10 promoter to associate with reversible curli expression, suggesting that various promoter mutations and mechanisms may induce constitutive curli expression.

To compare red versus white variant promoter strengths, we constructed *csgD*::*lacZ* promoter fusions of 43895OR, 43895OW, and 43894OR1 (17). Amplified products (using primers 5′-GGATCCACTTCATTAAACATGATGAAACCC and 5′-GCGCACCCAGTATTGTTA) were cloned into plasmid pCR2.1-TOPO (Invitrogen Corp., Carlsbad, Calif.), transferred into pMLB1034, expressed in *E. coli* strain DH5α, and tested for β-galactosidase specific activity (SA) (7). Although DH5α showed minimal Congo red binding following 48 h of growth at 28°C, we tested logarithmic-phase cultures grown in brain heart infusion at 37°C to eliminate any low-temperature or stationary-phase regulatory effects. Mean promoter strengths, calculated from three independent trials with two replicates per trial, were compared by one-way analysis of variance and Dunnett's two-tailed *t* test. The β-galactosidase activity was significantly greater for both red variants (43894OR, mean SA = 78,251, standard deviation [SD] = 748; 43895OR, mean SA = 64,127, SD = 13,519) compared to the white variant 43895OW (mean SA = 14,517, SD = 5,664; $P < 0.001$), demonstrating fourfold-greater red variant promoter expression. Regulatory factor differences between EHEC O157:H7 and K-12 strains or plasmid copy number effects could explain the higher-than-expected promoter expression from the curli-negative strain.

To determine the transforming effects of the red variant operon on the white variant strains, the *csgDEFG* operon and *csgB*-to-*csgD* intergenic region of 43894OR1 were amplified using primers COfor and PROrev and cloned into pCR2.1-TOPO to make plasmid pDEFG. Electrocompetent strains 43895OW, 43895FW, 43894OW1, 43894FWA, Tarr4A, Tarr1A,

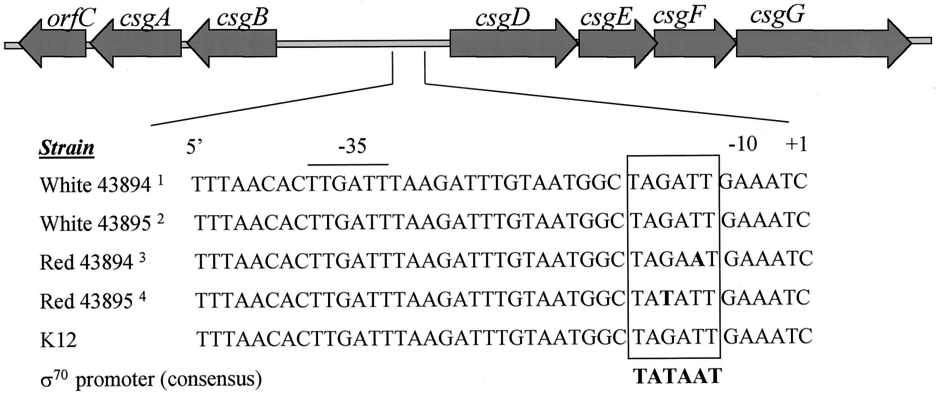

FIG. 2. Comparison of the DNA sequences of the intergenic region between *csgD* and *csgB* from 16 red and white variants of *E. coli* O157:H7 strains ATCC 43895 and ATCC 43894. The putative start of transcription of the *csgDEFG* operon is marked +1. The putative −10 promoter regions of variants are delineated with a box, and the −35 region is marked with a horizontal line. The base differences of red variants compared to white variants are shown in bold.

TABLE 1. *E. coli* strains transformed with pDEFG[a]

E. coli strain	Vector	Kanamycin resistance	Colony phenotype	Sequence of *csgD* promoter −10 on pDEFG
Top 10		−	Red	
	pCR2.1-TOPO	+	Red	
	pDEFG	+	Red	5′ TAGAAT
43895OW	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured[c]	−	White	
43895FW	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
43894OW	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
43894FW	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
84-2	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
161-2	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
Tarr1A	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	
Tarr4A	pCR2.1-TOPO	+	White	
	pDEFG	+	Red[b]	TAGATT
	Cured	−	White	

[a] pDEFG is pCR2.1-TOPO (Km^r) plus the recombinant *csgDEFG* operon of 43894OR1.
[b] Original transformed plates contained 90% red and 10% white colonies.
[c] Strains were cured of pDEFG by propagation on nonselective media.

84-2, and 161-2 were then transformed with pDEFG and recovered on CRI plates containing 50 μg of kanamycin/ml (16). We verified the presence of pDEFG by PCR using vector primer TOPOfor (5′-TGACCATGATTACGCCAAGC) and insert primer PROfor and sequenced the promoter region. All plasmid-transformed white strains produced approximately 90% red and 10% white colonies (Table 1). Red transformants contained pDEFG and were more mucoid than the parental 43894OR1 variant. However, sequencing revealed an unexpected adenosine-to-thymidine transversion at position −7 of the *csgD* promoter in all strains. Red transformants plated onto kanamycin-free media lost kanamycin resistance, reverted to the white phenotype and did not amplify a plasmid-specific 0.7-kb DNA fragment, indicating a loss of pDEFG. Strains transformed with pCR2.1-TOPO either containing no insert or containing the manufacturer's control insert produced smooth, moist, white colonies. The pDEFG-induced white-to-red variant switch and the red-to-white reversion in plasmid-cured transformants suggest *csgDEFG*-dependent phenotypic variation. A low ratio of normal (5′-TAGAAT) to transversion (5′-TAGATT) -bearing plasmids may explain the red phenotype of bacteria containing the white plasmid transversion.

TABLE 2. Substrate utilization of the red and white variants of *E. coli* O157:H7 strains ATCC 43895 and ATCC 43894[a]

Strain variant	Phenotype	Arginine	Pyruvate
43894OW1	White	−	−
43894OW2	White	−	−
43894OW3	White	−	−
43894FWA	White	−	−
43894FWB	White	−	−
43894FWC	White	−	−
43895OW	White	−	−
43895FW	White	−	−
43894OR1	Red	+	−
43894OR2	Red	+	+
43894OR3	Red	−	+
43894FRA	Red	+	+
43894FRB	Red	+	−
43894FRC	Red	−	+
43895OR	Red	+	−
43895FR	Red	−	+

[a] All 16 variants were identical in their utilization of the 30 other substrates measured by the Sensititre AP80 gram-negative autoidentification plate.

Comparison of substrate utilization by the 16 red and white (ATCC 43895 and ATCC 43894) variants by using Sensititre AP80 gram-negative autoidentification plates (AccuMed International Inc., Westlake, Ohio) showed identical usage patterns for 30 of 32 substrates (Table 2). However, all red variants uniquely utilized arginine and/or pyruvate, suggesting that *csgD* may influence gene expression beyond those involved in curli production.

These findings suggest that EHEC O157:H7 curli expression is uncommon but can occur in human strains in a temperature-independent phase-variant manner in association with *csg* promoter point mutations and with enhanced metabolic flexibility. The importance of curli expression in EHEC O157:H7 environmental survival and pathogenesis requires further investigation.

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